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H. 知的財産権の出願・登録状況

①特許取得（出願）

特許出願番号：特願2013-030455

発明の名称：潰瘍性大腸炎の予防または治療剤と新規フラーレン誘導体

出願国：日本

特許出願人:ビタミンC60 バイオリサーチ株式会社、国立大学法人大阪大学、増野匡彦、大江知之

発明者：堤 康央、吉岡靖雄、東阪和馬、増野匡彦、大江知之、山名修一、青島央江

出願年月日：2013 年 2 月 19 日

② 実用新案登録

該当無し

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<産官学アドバイザリーボード（外部評価）>

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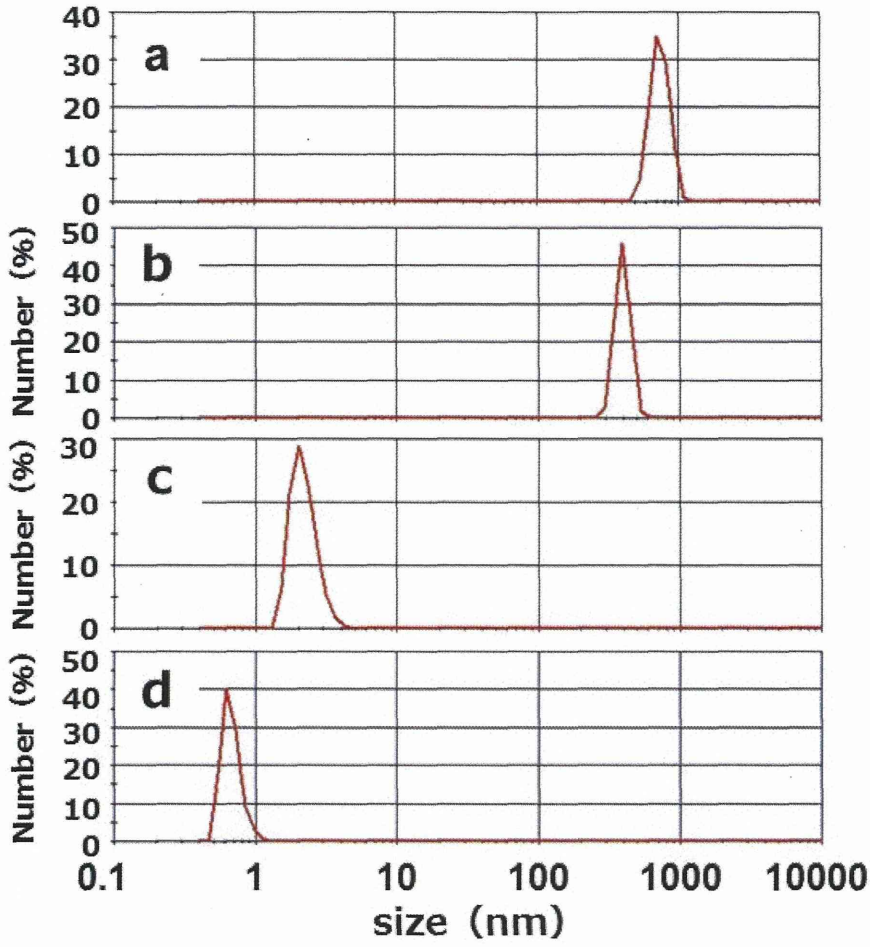


Fullerenol	$C_{60}(OH)_{12}$	$C_{60}(OH)_{24}$	$C_{60}(OH)_{36}$	$C60(OH)_{44}$
Mean particle size in solution (nm)	749	397	2.18	0.668

Fig 1. Particle size of Fullerenol particles. Fullerenol particles used in this study were suspended in distilled water and their sizesmeasured by dynamic light scattering (DSL). ($C_{60}(OH)_{12}$ (a), $C_{60}(OH)_{24}$ (b), $C_{60}(OH)_{36}$ (c), $C_{60}(OH)_{44}$ (d))

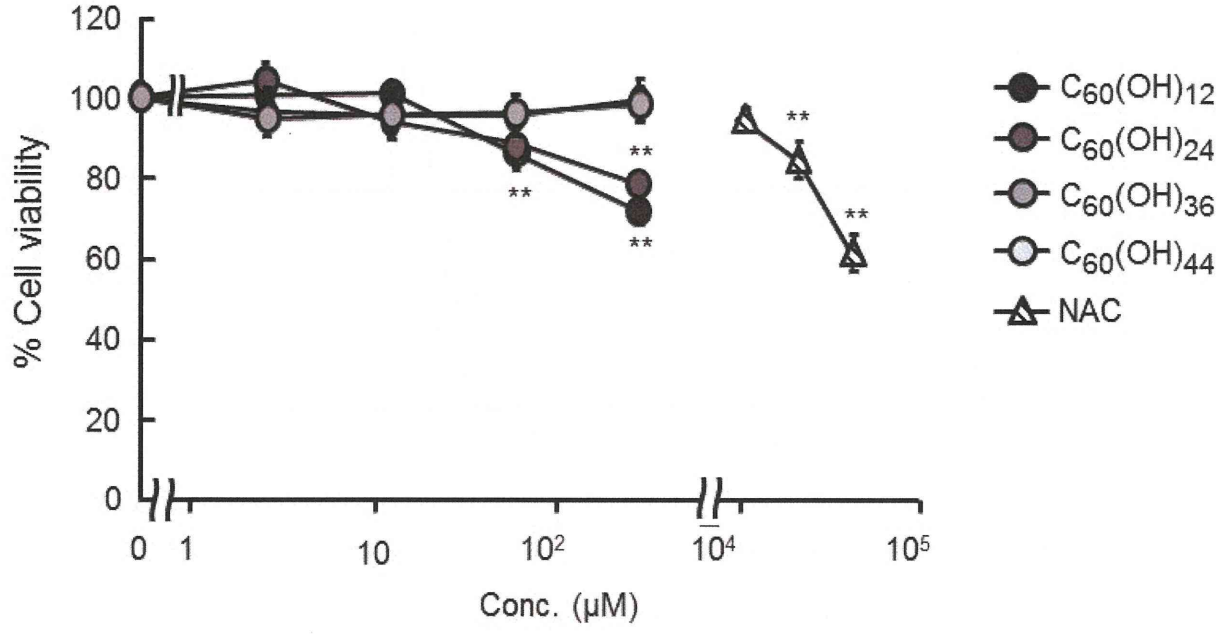


Fig 2. Cytotoxicity of Fullerenol particles in the Caco-2 cells. Caco-2 cells were treated with $C_{60}(OH)_{12}$, $C_{60}(OH)_{24}$, $C_{60}(OH)_{36}$, $C_{60}(OH)_{44}$ and NAC for 24 h. Cell viabilities were assessed by LDH Assay kit. Data are expressed as the mean ± S.D. (n = 6; **P < 0.01 versus value for control group by Tukey)

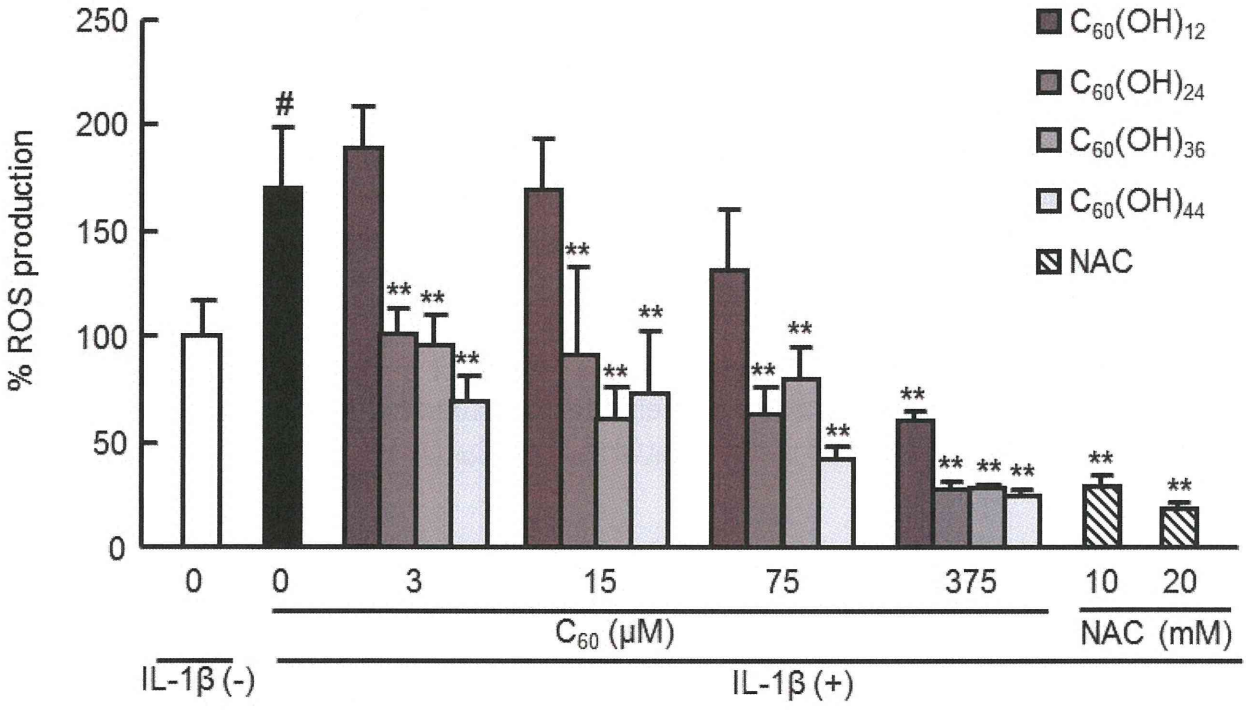


Fig 3. Inhibitory effects of Fullerenol particles on IL-1β-induced ROS production in the Caco-2 cells. ROS production was measured as DCF-fluorescence intensity. Caco-2 cells were pretreated with 20 μM of DCFH-DA for 30 min then treated with Fullerenol particles or NAC for 30 min, and then, stimulated with IL-1β (125 ng/ml) for 24 h. Data are expressed as the mean ± S.D. (n = 6; $^{\#}P < 0.01$ versus value for control group by Tukey ; $^{**}P < 0.01$, $^{*}P < 0.05$ versus value for IL-1β group by Tukey)

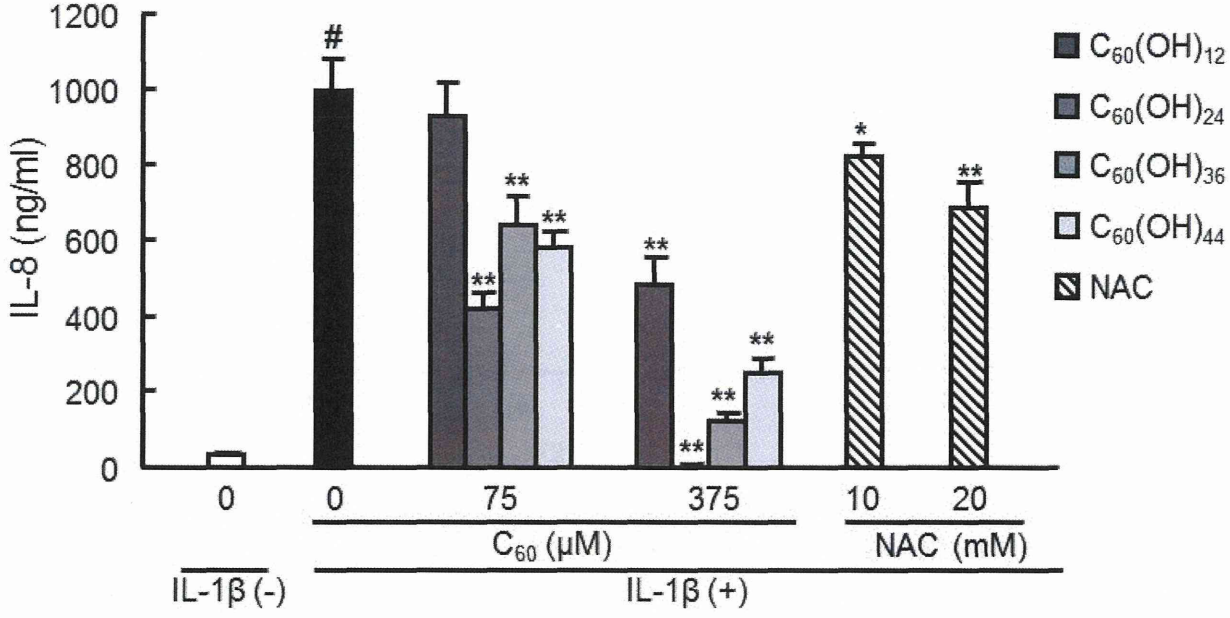


Fig 4. Inhibitory effects of Fullerenol particles on IL-1β-induced IL-8 secretion in the Caco-2 cells. Caco-2 cells were treated with Fullerenol particles or NAC for 30 min, and then, stimulated with IL-1β (125 ng/ml) for 24 h. Secreted IL-8 protein level in the culture supernatant was measured by IL-8 ELISA kit. Data are expressed as the mean ± S.D. (n = 6; $^{\#}P < 0.01$ versus value for control group by Tukey ; $^{**}P < 0.01$, $^{*}P < 0.05$ versus value for IL-1β group by Tukey)

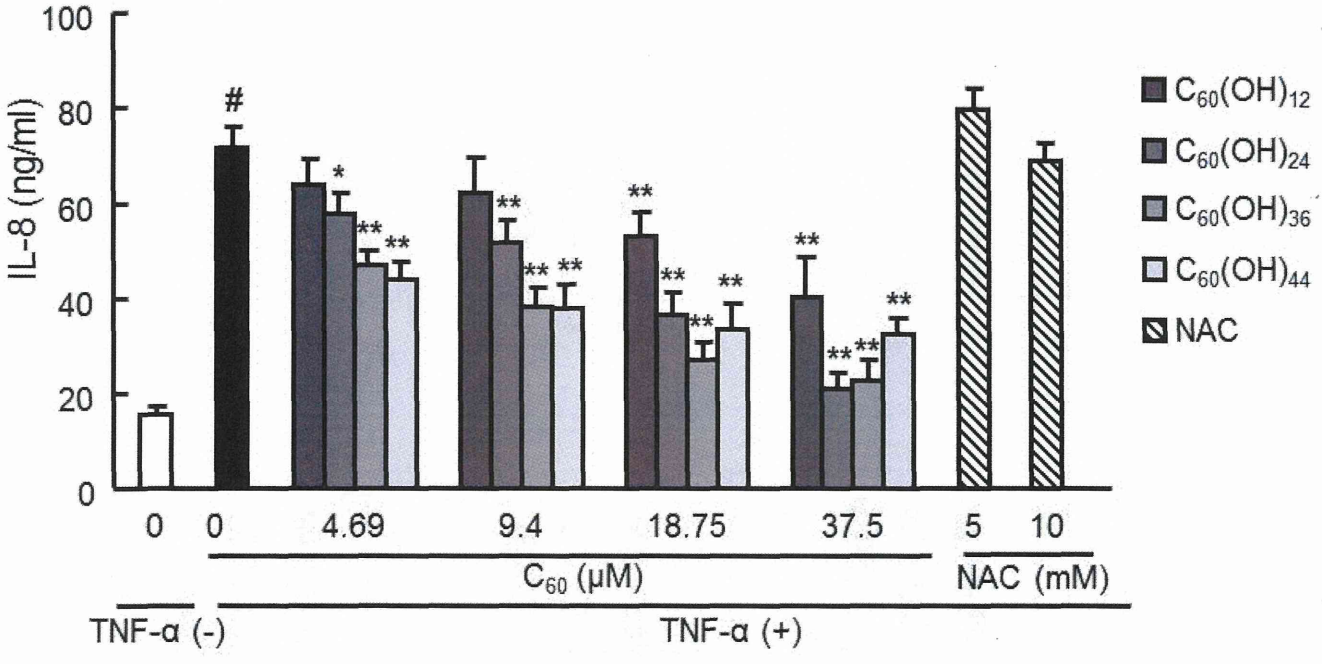


Fig 5. Inhibitory effects of Fullerenol particles on TNF-α-induced IL-8 secretion in the Caco-2 cells. Caco-2 cells were treated with Fullerenol particles or NAC for 30 min, and then, stimulated with TNF-α (10 ng/ml) for 24 h. Secreted IL-8 protein level in the culture supernatant was measured by IL-8 ELISA kit. Data are expressed as the mean ± S.D. (n = 6; $^{\#}P < 0.01$ versus value for control group by Tukey ; $^{**}P < 0.01$, $^{*}P < 0.05$ versus value for TNF-α group by Tukey)

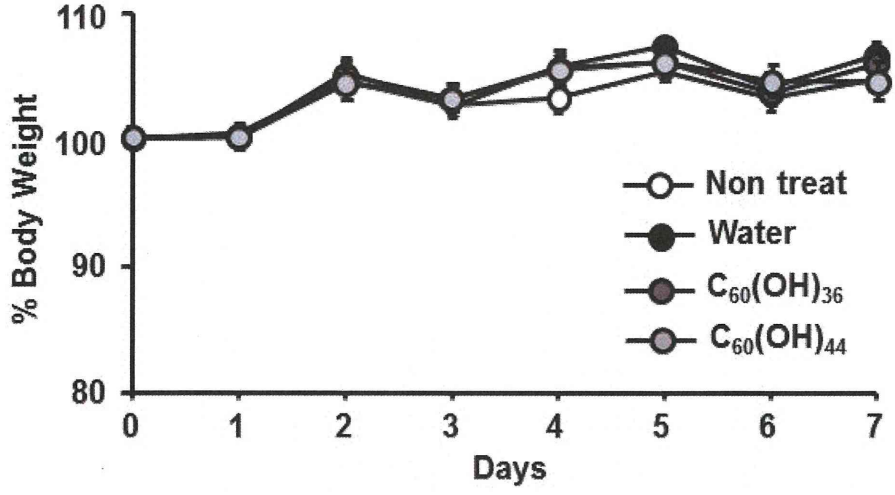


Fig 6. Changes of body weight of mice treated by Fullerenol particles. Fullerenol particles were administered p.o. daily for 7 days. Data are expressed as the mean ± S.E. (n=6).

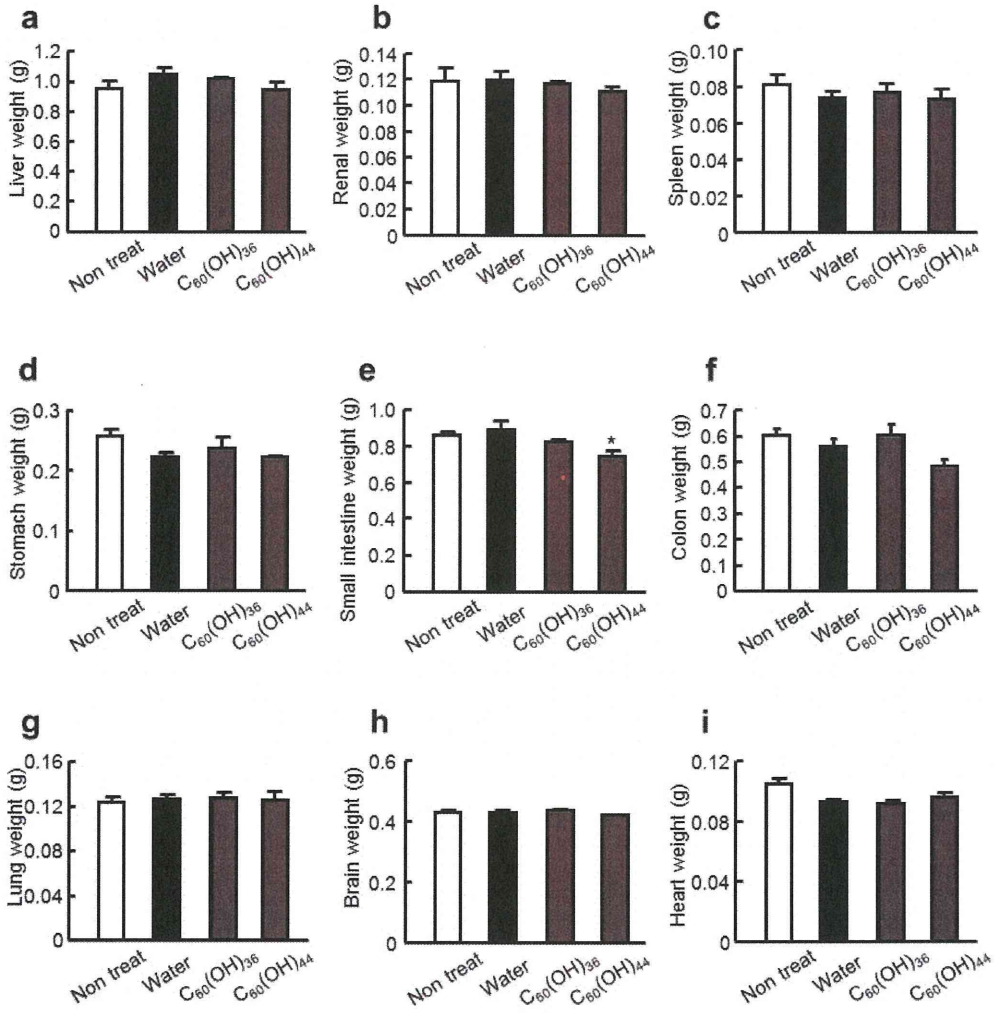


Fig 7. Changes of organ weight of mice treated by Fullerenol particles Fullerenol particles or water were administered p.o. daily for 7 days. The wet weight of organ (liver (a), renal (b), spleen (c), stomach (d), small intestine (e), colon (f), lung (g), brain (h), heart (i)) were measured. Data are expressed as the mean ± S.E. (n = 6; $^{**}P < 0.01$, $^{*}P < 0.05$ versus value for control (water) group by Tukey)

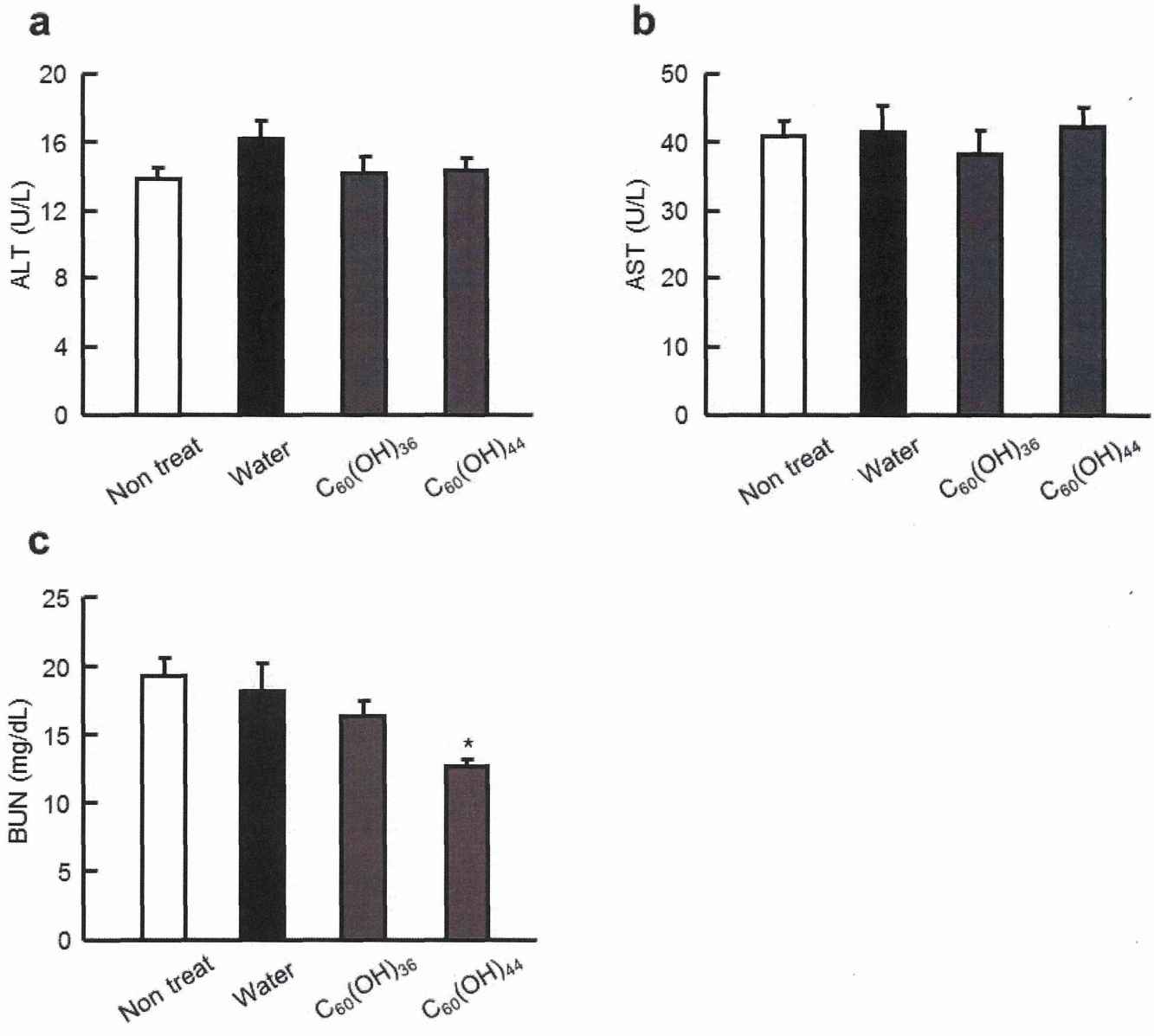


Fig 8. Changes of biochemical parameters in blood of mice treated by Fullerenol particles. Blood samples were collected from BALB/c mice was oral administered Fullerenol particles or water at 24 h after the last exposure for 7 days. Plasma was recovered by contrifuging blood at 1750 ´ g for 15 min. Biochemical parameters were analyzed using FUJI DRI-CHEM 7000 (ALT (a), AST (b), BUN (c)). Data are expressed as the mean ± S.E. (n = 6; $^{**}P < 0.01$, $^{*}P < 0.05$ versus value for control (water) group by Tukey).

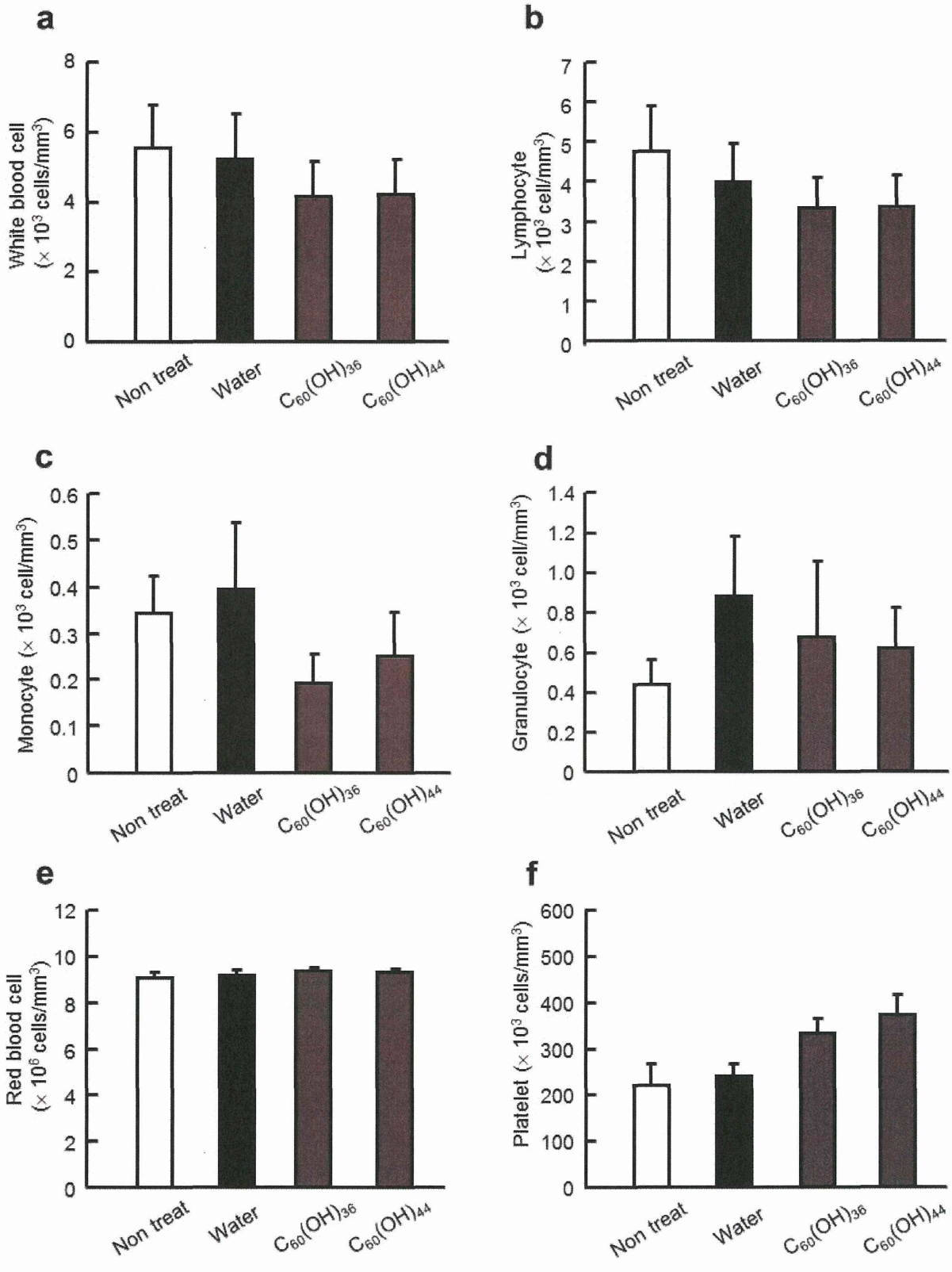


Figure 9. Changes of hematological parameters in blood of mice treated by Fullerenol particles. Blood samples were collected from BALB/c mice was oral administered Fullerenol particles or water at 24 h after the last exposure for 7 days. Hematology analysis was performed using VetScan (white blood cells (a), lymphocytes (b), Granulocytes (c), monocytes (d), red blood cells (e), platelets (f)). Data are expressed as the mean ± S.E. (n = 6; $^{**}P < 0.01$, $^{*}P < 0.05$ versus value for control (water) group by Tukey).

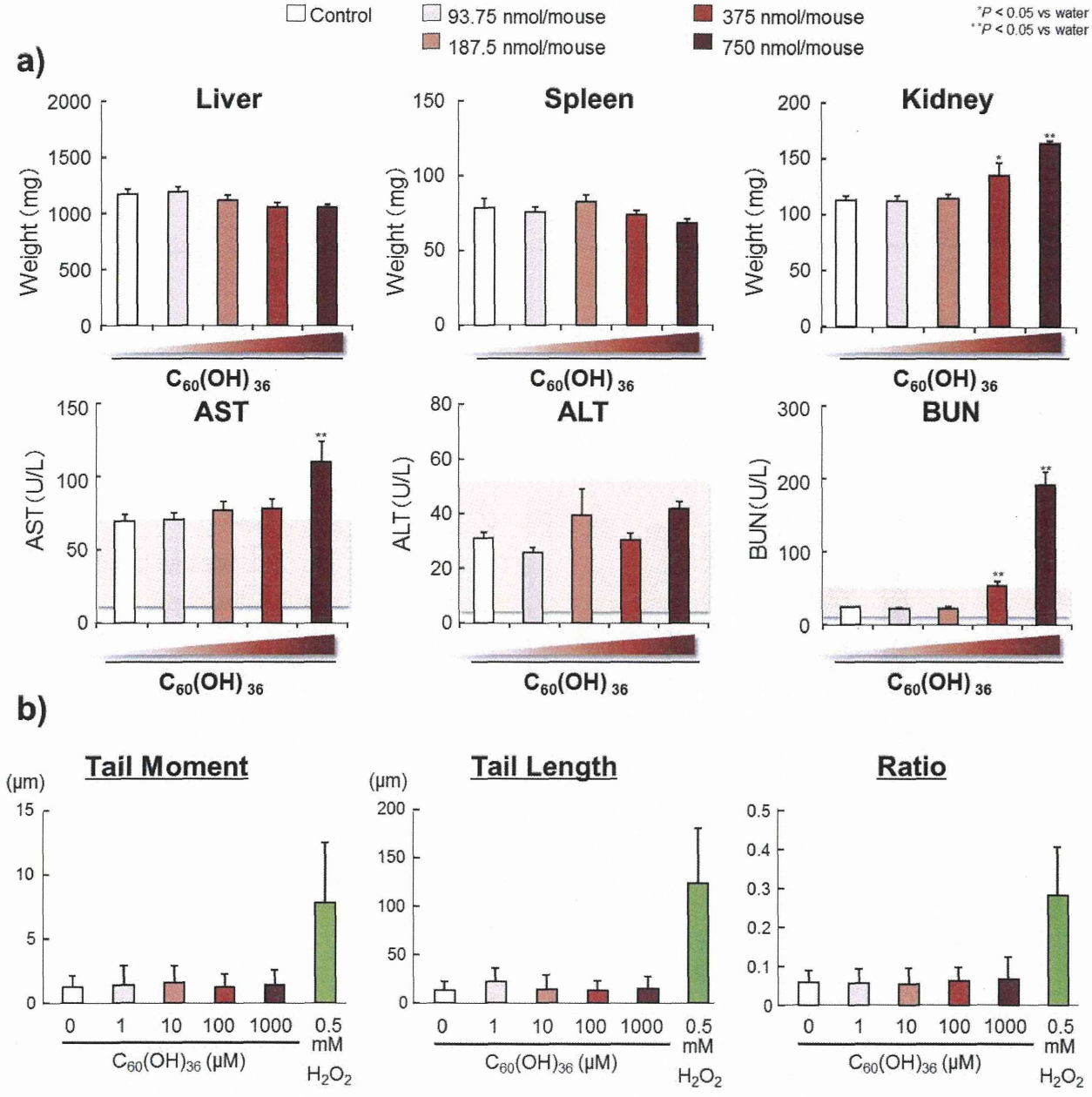


Fig 10. Changes of biochemical parameters in blood of mice treated by Fullerenol particles. a) Blood samples were collected from BALB/c mice treated with Fullerenol particles or water at 24 h intravenously. Plasma was recovered by contrifuging blood at 1750 ´ g for 15 min. Biochemical parameters were analyzed using FUJI DRI-CHEM 7000 Data are expressed as the mean ± S.E. (n = 6; $^{**}P < 0.01$, $^{*}P < 0.05$ versus value for control (water) group by Tukey). b) Caco-2 cells were treated with 0.2 mM H2O2 or Fullerenol particles. DNA strand breaks were detected by alkaline comet assay according to the Comet Assay Kit

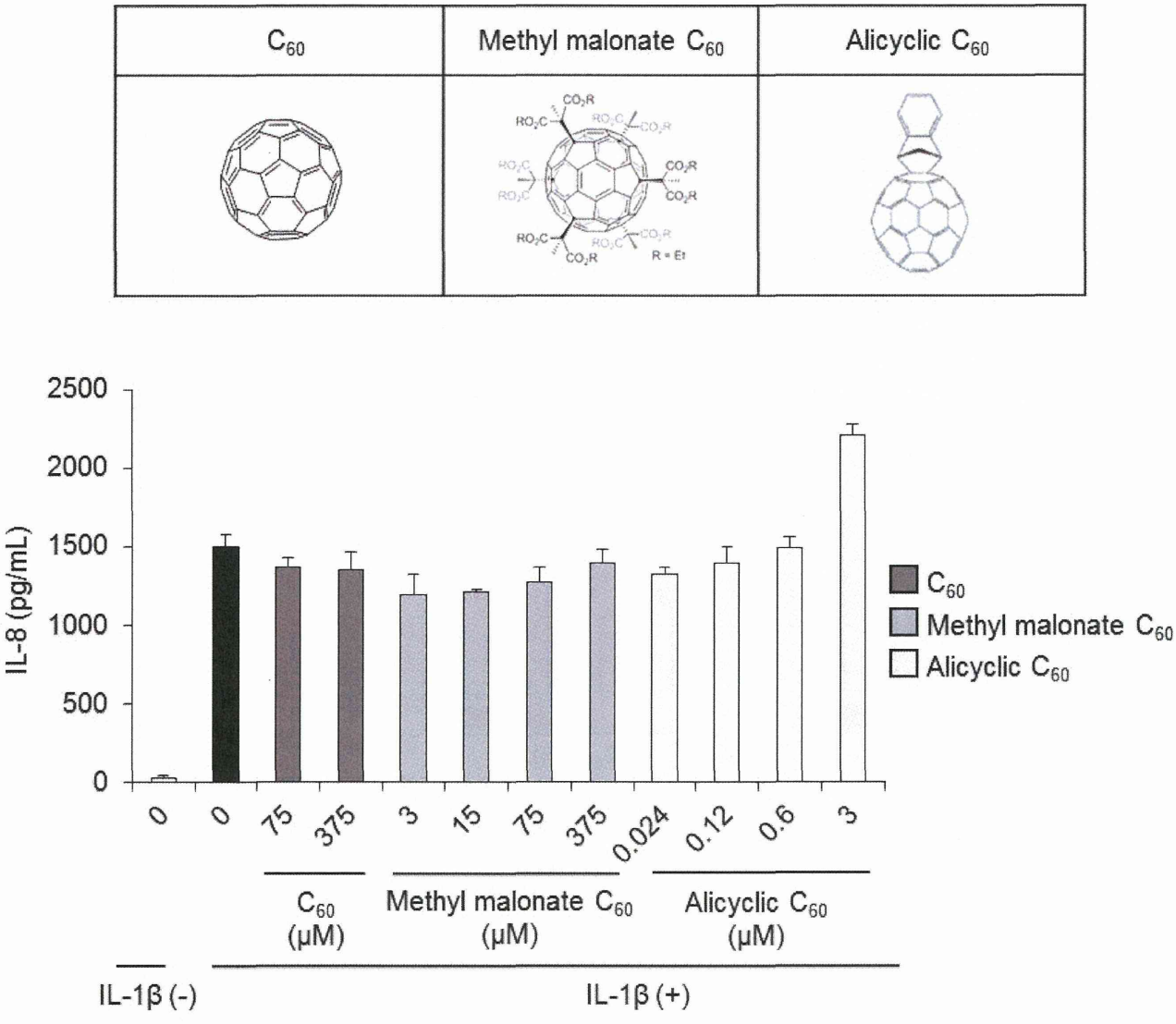


Fig 11. Inhibitory effects of fullerene C_{60} derivatives on IL-1β-induced IL-8 secretion in the Caco-2 cells. Caco-2 cells were treated with fullerene C_{60} or fullerene C_{60} derivatives for 30 min, and then, stimulated with IL-1β (125 ng/ml) for 24 h. Secreted IL-8 protein level in the culture supernatant was measured by IL-8 ELISA kit. Data are expressed as the mean ± S.D. (n = 4)

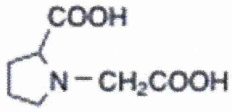
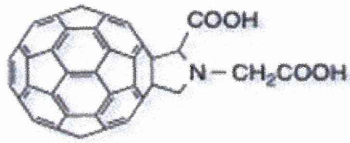
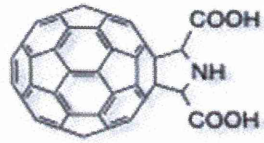
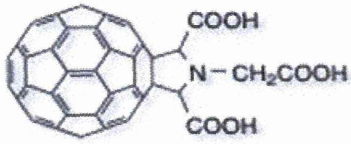
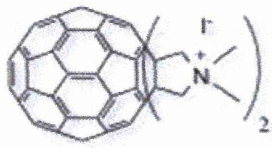
Proline (1)'	Proline C_{60} (1)	
		
Proline C_{60} (3)	**Proline C_{60} (4)**	**$C_{60}(OH)_{36}$**
		

Fig 12. The chemical structure formula of proline-modified fullerene C_{60} derivatives, functional group of proline $C_{60}(1)$ and $C_{60}(OH)_{36}$.

	Particle size In solution (nm)	Zeta potential (mV)
Proline C_{60} (1)	114 (608)	-26.5
Proline C_{60} (2)	152 (629)	-29.2
Proline C_{60} (3)	32.6	-17.1
Proline C_{60} (4)	52.7	+40.0
$C_{60}(OH)_{36}$	2.1	-28.9
C_{60}	338	-14.0

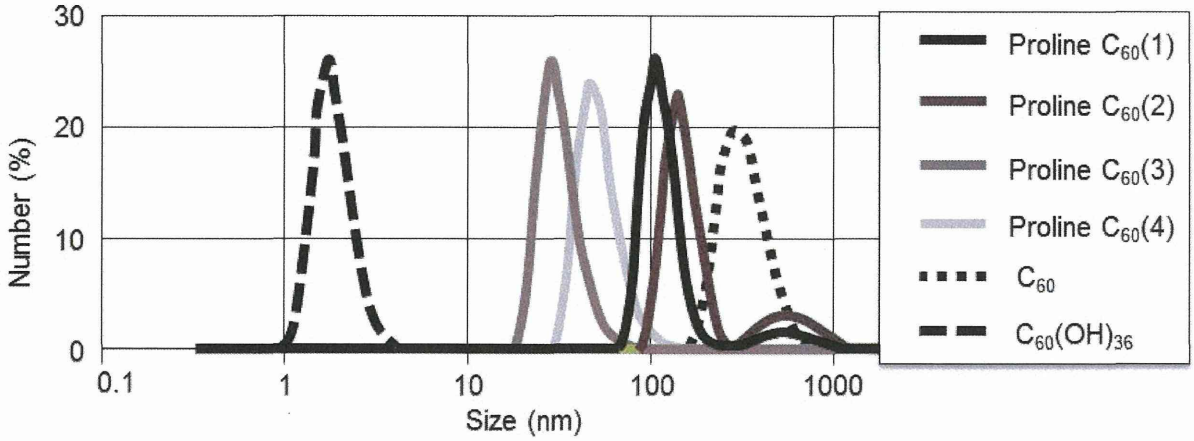


Fig 13. Particle size of proline-modified fullerene C_{60} derivatives. Proline-modified fullerene C_{60} derivatives used in this study were suspended in DMSO and then suspended in distilled water and their sizes (proline C_{60} (1), proline C_{60} (2), proline C_{60} (3), proline C_{60} (4), C_{60} or $C_{60}(OH)_{36}$) were measured by dynamic light scattering (DSL).

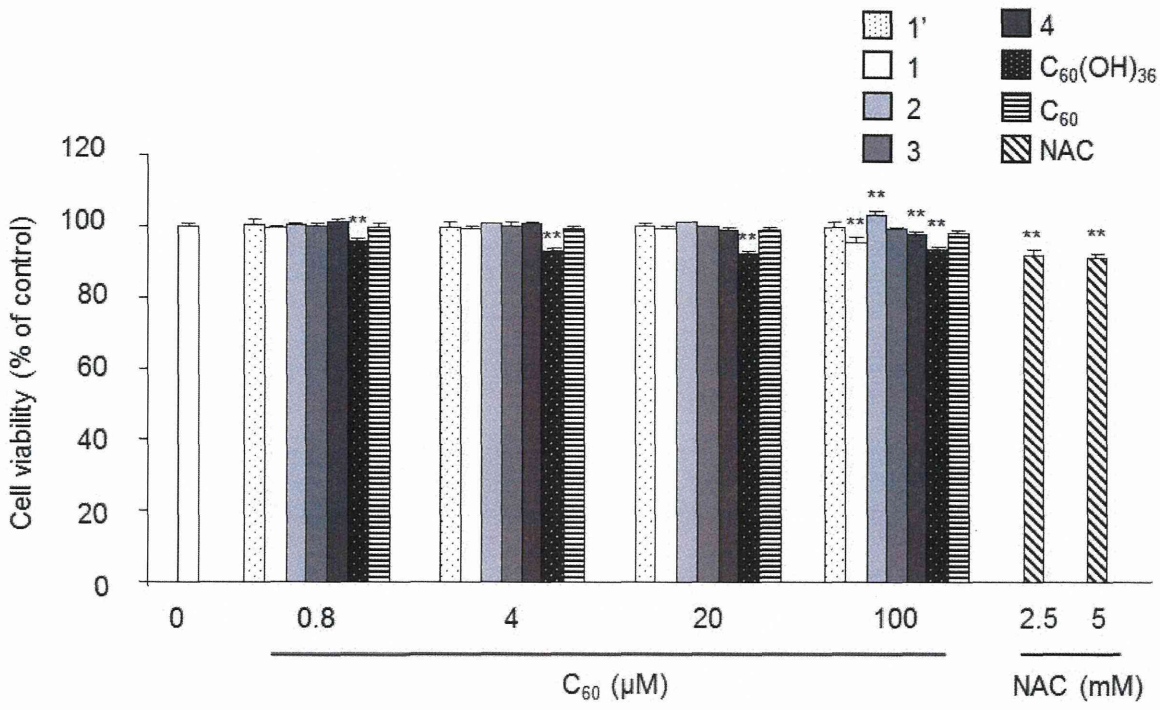


Fig 14. Cytotoxicity of proline-modified fullerene C_{60} derivatives in the Caco-2 cells. Caco-2 cells were treated with proline-modified fullerene derivatives, $C_{60}(OH)_{36}$, C_{60} and NAC for 24 h. Cell viabilities were assessed by LDH Assay kit. Data are expressed as the mean ± S.D. (n = 4; $^{**}P < 0.01$ versus value for control group by Bonferroni)

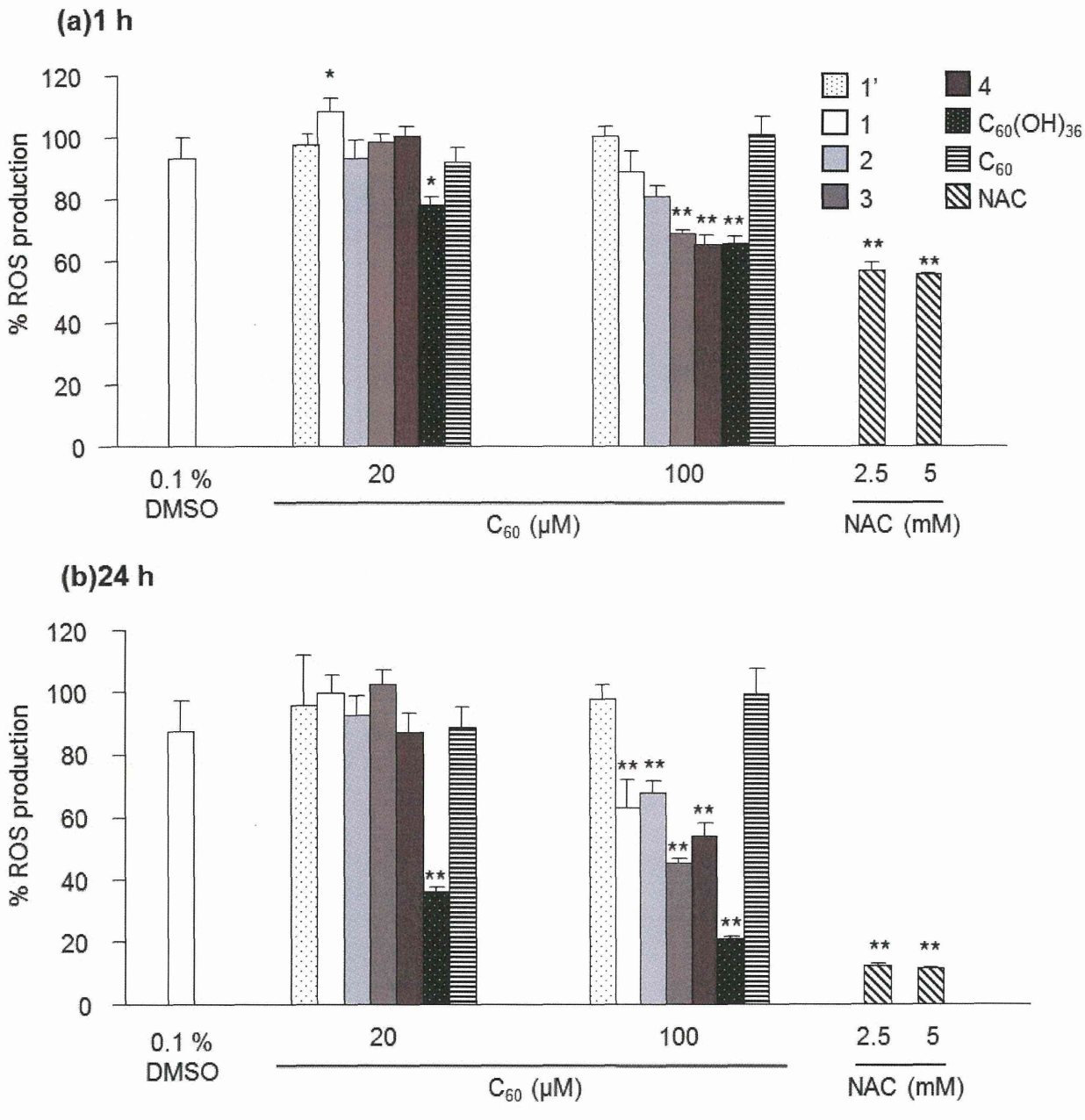


Fig 15. Inhibitory effects of proline-modified fullerene C_{60} derivatives on intracellular ROS production in the Caco-2 cells. Intracellular ROS production was measured as DCF-fluorescence intensity. Caco-2 cells were treated with 20 μM of DCFH-DA for 20 min then treated with proline-modified fullerene C_{60} derivatives, $C_{60}(OH)_{36}$, C_{60} or NAC for 1 h (a) or 24 h (b). Data are expressed as the mean ± S.D. (n = 4; $^{**}P < 0.01$, $^{*}P < 0.05$ versus value for 0.1 % DMSO group by Bonferroni)

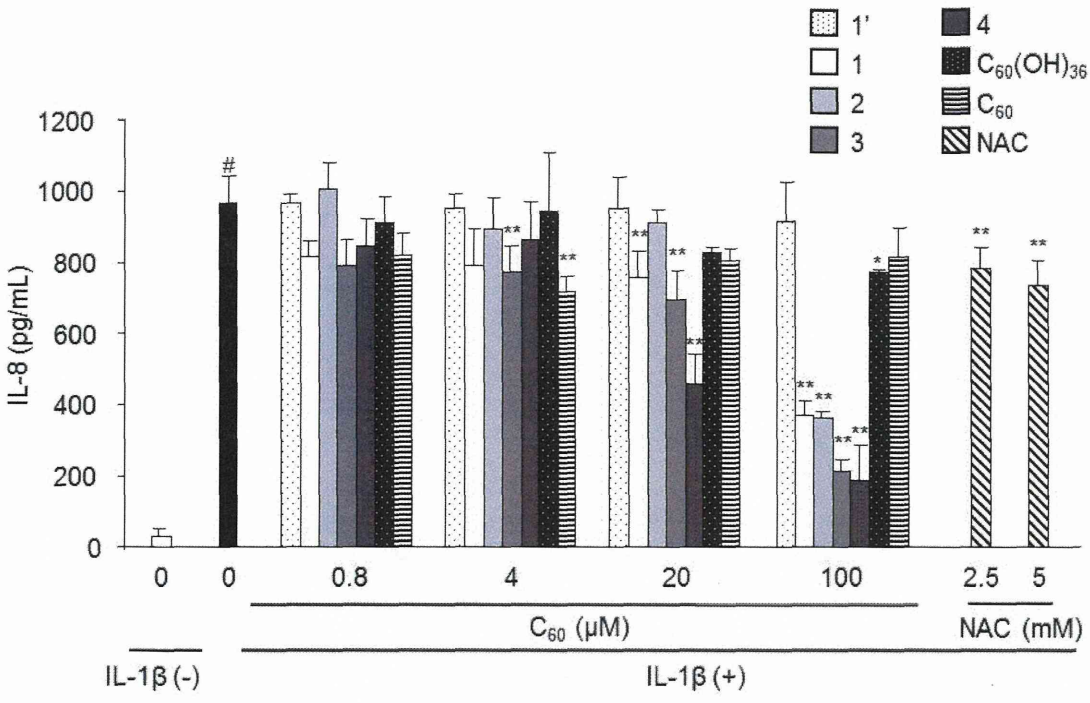


Fig 16. Inhibitory effects of proline-modified fullerene C_{60} derivatives on IL-1β-induced IL-8 secretion in the Caco-2 cells. Caco-2 cells were treated with proline-modified fullerene C_{60} derivatives, $C_{60}(OH)_{36}$, C_{60} or NAC for 30 min, and then, stimulated with IL-1β (125 ng/ml) for 24 h. Secreted IL-8 protein level in the culture supernatant was measured by IL-8 ELISA kit. Data are expressed as the mean ± S.D. (n = 4; $^{\#}P < 0.01$ versus value for control group by Bonferroni ; $^{**}P < 0.01$, $^{*}P < 0.05$ versus value for IL-1β group by Bonferroni)

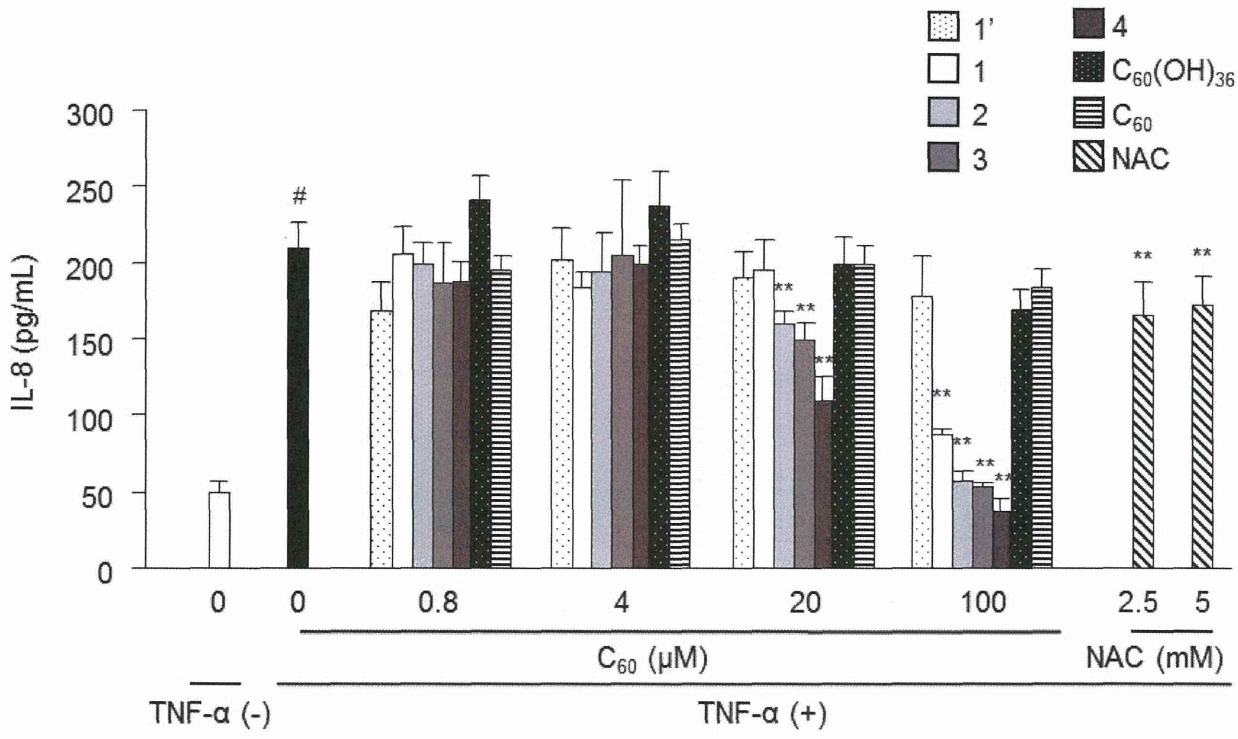


Fig 17. Inhibitory effects of proline-modified fullerene C_{60} derivatives on TNF-α-induced IL-8 secretion in the Caco-2 cells. Caco-2 cells were treated with proline-modified fullerene C_{60} derivatives, $C_{60}(OH)_{36}$, C_{60} or NAC for 30 min, and then, stimulated with TNF-α (10 ng/ml) for 24 h. Secreted IL-8 protein level in the culture supernatant was measured by IL-8 ELISA kit. Data are expressed as the mean ± S.D. (n = 4; $^{\#}P < 0.01$ versus value for control group by Bonferroni ; $^{**}P < 0.01$, $^{*}P < 0.05$ versus value for TNF-α group by Bonferroni)

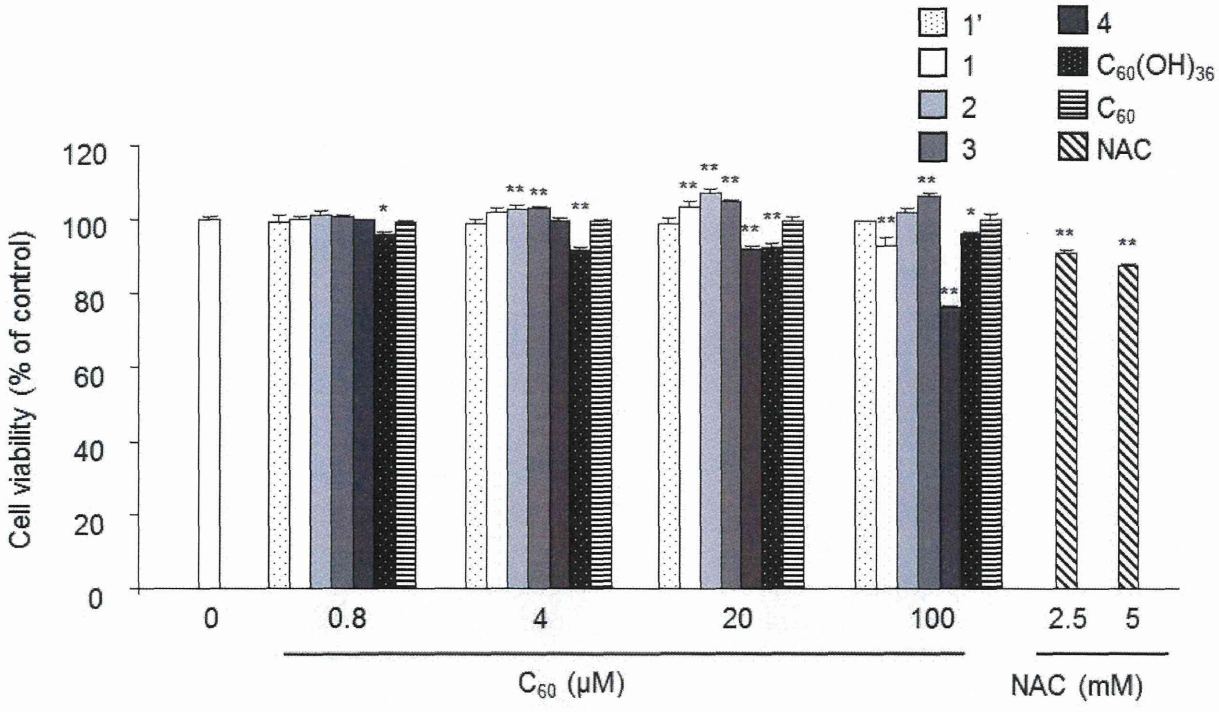


Fig 18. Cytotoxicity of proline-modified fullerene C_{60} derivatives in the RAW264.7 cells. RAW264.7 cells were treated with proline-modified fullerene derivatives, $C_{60}(OH)_{36}$, C_{60} and NAC for 24 h. Cell viabilities were assessed by LDH Assay kit. Data are expressed as the mean ± S.D. (n = 4; **P < 0.01 versus value for control group by Bonferroni)

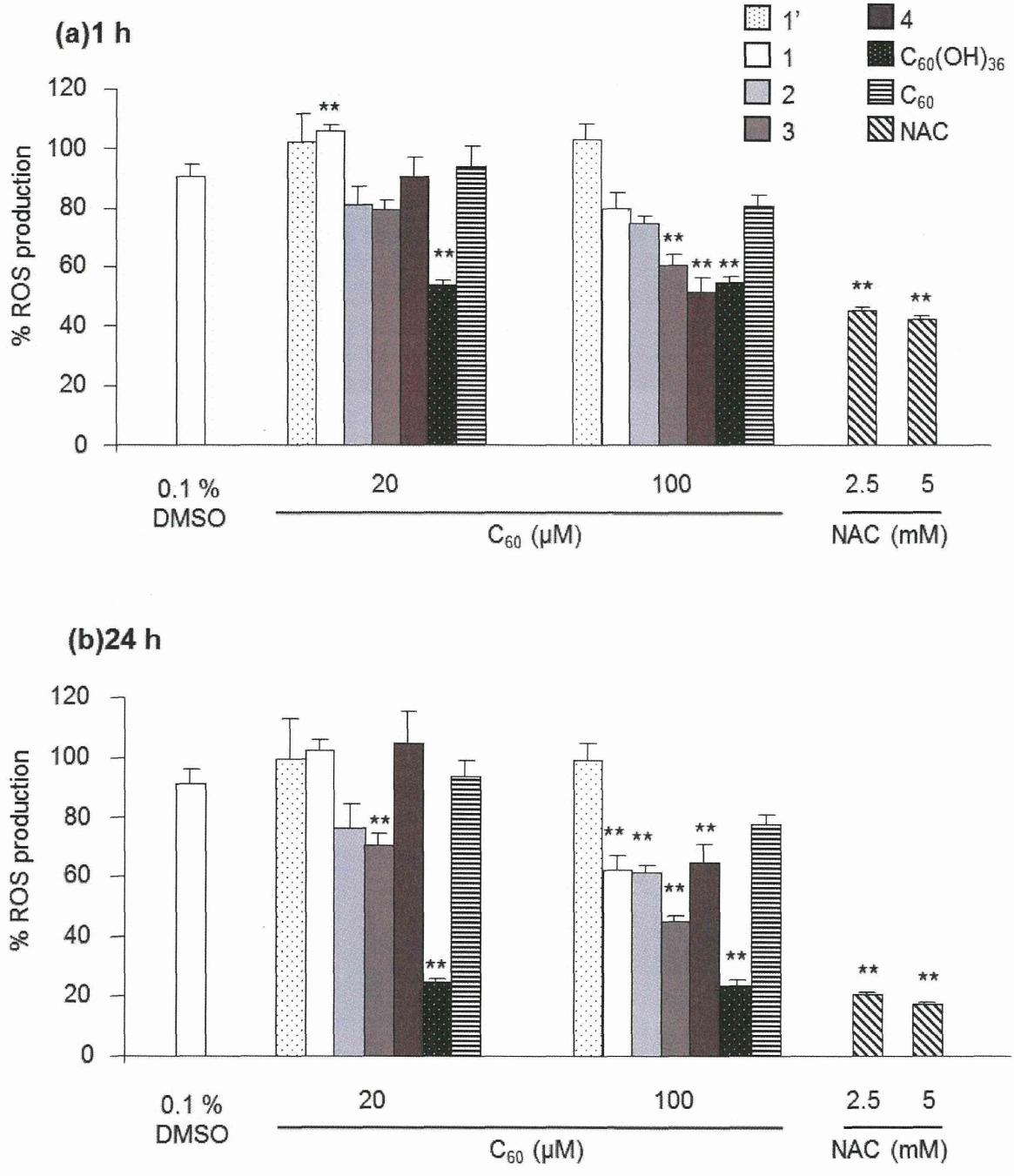


Fig 19. Inhibitory effects of proline-modified fullerene C_{60} derivatives on intracellular ROS production in the RAW264.7 cells. Intracellular ROS production was measured as DCF-fluorescence intensity. RAW264.7 cells were treated with 20 μM of DCFH-DA for 20 min then treated with proline-modified fullerene C_{60} derivatives, $C_{60}(OH)_{36}$, C_{60} or NAC for 1 or 24 h. Data are expressed as the mean ± S.D. (n = 4; $^{**}P < 0.01$, $^{*}P < 0.05$ versus value for 0.1 % DMSO group by Bonferroni)

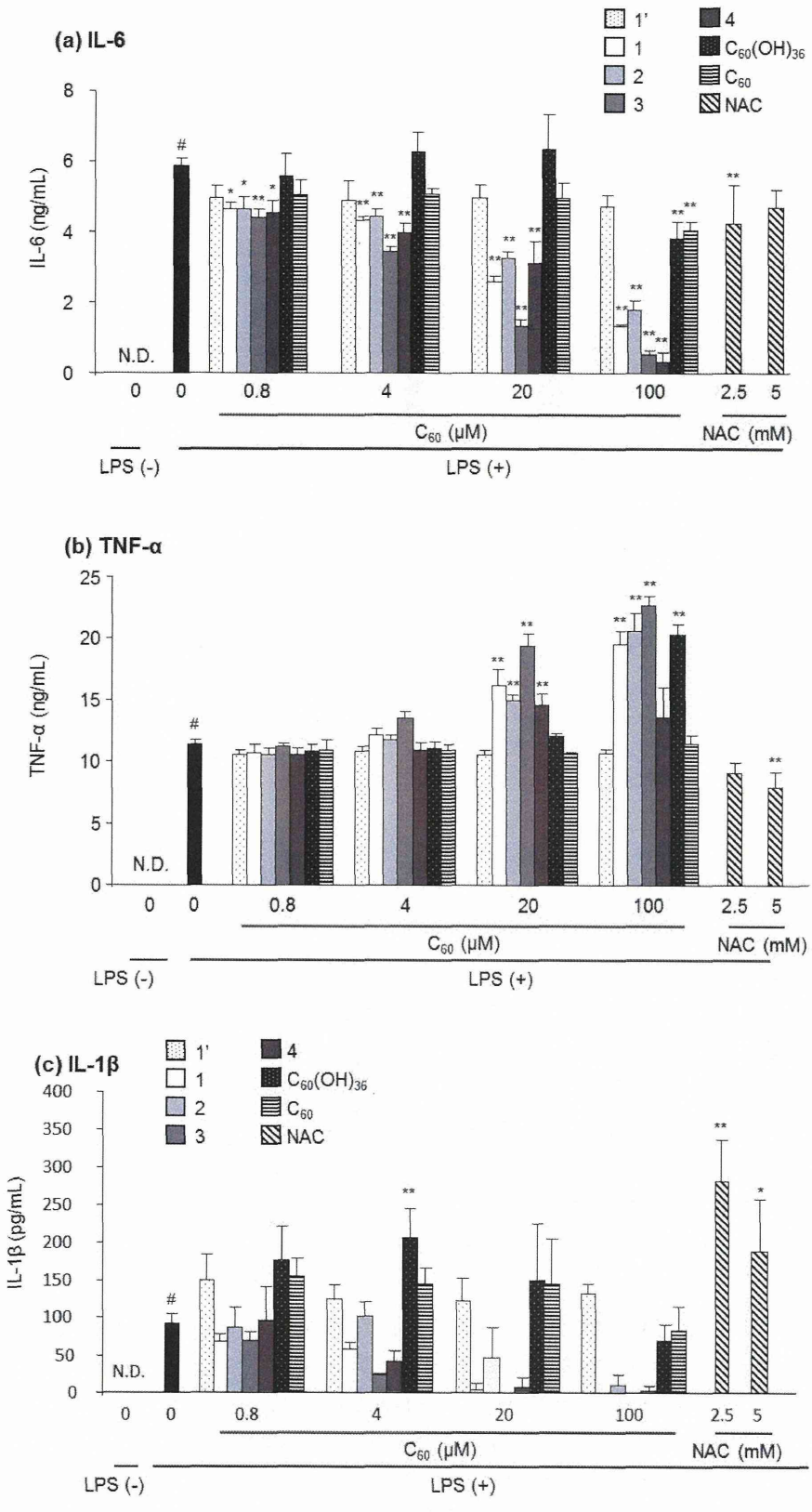


Fig 20. Inhibitory effects of proline-modified fullerene C_{60} derivatives on LPS-induced IL-6, TNF-α, or IL-1β secretion in the RAW264.7 cells. RAW264.7 cells were treated with proline-modified fullerene C_{60} derivatives, $C_{60}(OH)_{36}$, C_{60} or NAC for 30 min, and then, stimulated with LPS (1 μg/ml) for 24 h. Secreted IL-6 (a), TNF-α (b), IL-1β (c) protein level in the culture supernatant was measured by IL-6, TNF-α, IL-1β, IFN-β or IP-10 ELISA kit. Data are expressed as the mean ± S.D. (n = 4; $^{\#}P <$ 0.05 versus value for control group by Bonferroni ; $^{**}P < 0.01$, $^{*}P < 0.05$ versus value for LPS group by Bonferroni)

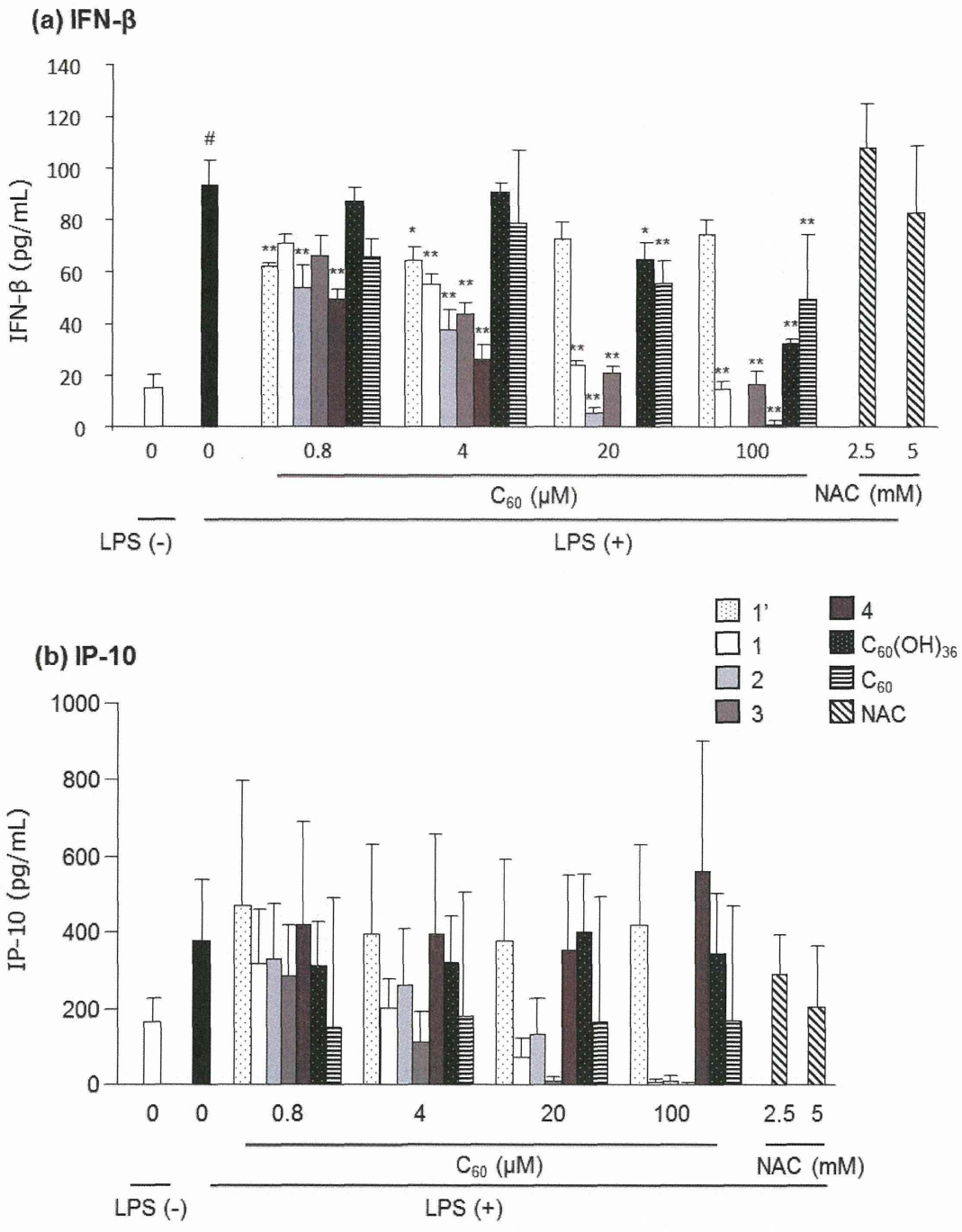


Fig 21. Inhibitory effects of proline-modified fullerene C_{60} derivatives on LPS-induced IFN-β or IP-10 secretion in the RAW264.7 cells. RAW264.7 cells were treated with proline-modified fullerene C_{60} derivatives, $C_{60}(OH)_{36}$, C_{60} or NAC for 30 min, and then, stimulated with LPS (1 μg/ml) for 24 h. Secreted IFN-β (a) or IP-10 (b) protein level in the culture supernatant was measured by IL-6, TNF-α, IL-1β, IFN-β or IP-10 ELISA kit. Data are expressed as the mean ± S.D. (n = 4; $^{\#}P < 0.05$ versus value for control group by Bonferroni ; $^{**}P < 0.01$, $^{*}P < 0.05$ versus value for LPS group by Bonferroni)